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Molecular and morphological differentiation between *Aphis gossypii* Glover (Hemiptera, Aphididae) and related species, with particular reference to the North American Midwest

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Abstract

The cotton aphid, *Aphis gossypii*, is one of the most biologically diverse species of aphids; a polyphagous species in a family where most are host specialists. It is economically important and belongs to a group of closely related species that has challenged aphid taxonomy. The research presented here seeks to clarify the taxonomic relationships and status of species within the *A. gossypii* group in the North American Midwest. Sequences of the mitochondrial cytochrome oxidase 1 (COI), nuclear elongation factor 1-α (EF1-α), and nuclear sodium channel para-type (SCP) genes were used to differentiate between *A. gossypii* and related species. *Aphis monardae*, previously synonymised with *A. gossypii*, is re-established as a valid species. Phylogenetic analyses support the close relationship of members of the *A. gossypii* group native to North America (*A. forbesi*, *A. monardae*, *A. oestlundi*, *A. rubifolii*, and *A. rubicola*), Europe (*A. nasturtii*, *A. urticata* and *A. sedi*), and Asia (*A. agrimoniae, A. clerodendri, A. glycines, A. gossypii, A. hypericiphaga, A. ichigicola, A. ichigo, A. sanguisorbicola, A. sumire* and *A. taraxicicola*). The North American species most closely related to *A. gossypii* are *A. monardae* and *A. oestlundi*. The cosmopolitan *A. gossypii* and *A. sedi* identified in the USA are genetically very similar using COI and EF1-α sequences, but the SCP gene shows greater genetic distance between them. We present a discussion of the biological and morphological differentiation of these species.

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Keywords
Aphid, host plant, morphology, phylogeny, sequence divergence, status novus

Introduction

Host plant association is often one of the main characters used to distinguish between closely related aphid species. However, host association can also be one of the main sources of misidentification of host-alternating aphids. These aphids migrate between taxonomically distant hosts, usually between woody and herbaceous plants. Taxonomic problems have been created when aphid morphs from primary (woody) host plants have been treated as separate species from those found living on secondary (herbaceous) or summer host plants. Host alternation provides an opportunity for aphids to acquire new hosts and may be a key to the rapid diversification of some groups of aphids (Eastop 1971, Dixon 1973, von Dohlen and Moran 2000), thereby leading to hard-to-distinguish species complexes. The evolution of *Aphis*, the largest aphid genus by a margin, is associated with the rapid diversification of herbaceous angiosperms (Heie 1996).

The *Aphis gossypii* group contains economically important and taxonomically problematic species, with *A. gossypii* Glover itself being the most biologically diverse and hence taxonomically challenging (Blackman and Eastop 2007). It has many different primary and secondary host plants and exhibits both holocyclic and anholocyclic life cycles (Kring 1955, Blackman and Eastop 2006, Margaritopoulus et al. 2006). Its taxonomic complexity is attested to by its 42 available synonyms, including the native North American species, *Aphis monardae* Oestlund (Favret 2014). Eastop and Hille Ris Lambers (1976) established this synonymy without comment. Lagos (2007) found that *A. monardae* is distinct morphologically from *A. gossypii* and treated it as a valid species. No type specimen of *A. monardae* could be found at the time, and no molecular or biological evidence was available to support this decision. The research presented here contains both molecular and biological evidence as well as an examination of material collected by Oestlund from *Monarda* spp.

In Europe, there are approximately 20 aphid species morphologically similar to *A. gossypii* (Stroyan 1984, Heie 1986). Several studies using mitochondrial, nuclear, and intron length polymorphism in the sodium channel para-type (SCP) genes have achieved some resolution discriminating *A. gossypii* and other *Aphis* species (Coeur d'acier et al. 2007, Foottit et al. 2008, Coccuzza et al. 2009, Carletto et al. 2009b, Kim et al. 2010a, Komazaki et al. 2010, Favret and Miller 2011). In North America, morphological studies show that species of the *A. gossypii* group can be misidentified easily (Voegtlin et al. 2004, Lagos 2007). The discrimination of species closely related to *A. gossypii* is of particular importance due to the recent introduction into North America of the soybean aphid, *A. glycines* Matsumura (Voegtlin et al. 2004). This species is obligately holocyclic and heteroecious, feeding on soybean, *Glycine max* (L.) Merr., as secondary host, and on *Rhamnus* spp. as primary host. *Aphis gossypii* has also

been reported to colonize soybean in North America (Blackman and Eastop 2007), and while its colonization on soybeans is uncommon in the north central United States, some soybean-collected insect samples from Alabama, Georgia, Kansas, Louisiana, and Mississippi contained only *A. gossypii* or a mixture of both species (personal observation, Illinois Natural History Survey (INHS) insect collection records). These collections suggest that *A. gossypii* may be more common on soybeans in southern regions. There are no records of the exotic *A. nasturtii* Kaltenbach feeding on soybeans, and attempts to culture *A. nasturtii* on soybeans were not successful (David Ragsdale, personal communication); however, this species shares the primary host, *Rhamnus* spp., with both *A. glycines* and *A. gossypii*.

We here elucidate the phylogenetic relationship of species morphologically close to *A. gossypii* and the taxonomic status of *A. monardae* in the North American Midwest.

Materials and methods

Aphid collections: Aphids were collected from their primary and/or secondary host plants from different sites in China, France, Italy, Japan, Spain and the USA, with the majority of the material originating from the Midwest of the USA. When possible, aphids were collected alive and reared on the host plant for the maturation of late instar nymphs. Adults were preserved in 95% ethanol and stored at -20°C until DNA extraction and microscope slide preparation. Collection data with INHS Insect Collection specimen voucher numbers are presented in Suppl. material 1.

Morphology: Archival microscope slides were prepared using the technique described by Pike et al. (1991). Individuals were selected from the same colonies as those selected for DNA extraction. Photographs of mounted specimens and measurements were taken using a Leica DM 2000 digital camera and SPOT Software 4.6 (Diagnostic Instruments, Inc). Analyses of variance of diagnostic characters, such as the distance from the base of the third antennal segment to the first secondary sensorium, the ratio of the lengths of the processus terminalis and the base of the sixth antennal segment, and the ratio of the lengths of the siphunculus and the cauda, were tested using JMP, Version 7 (SAS Institute Inc., Cary, NC, 1989–2007). Species identification of slide-mounted material was done by the first author, using published keys (Oestlund 1887, Gillette 1927, Hottes and Frison 1931, Palmer 1952, Kring 1955, Cook 1984, Voegtlin et al. 2004) and authoritatively identified specimens in the insect collections of the INHS and the University of Minnesota. Identifications of slide-mounted specimens were referenced to the aphid colony-mates used in the molecular analyses.

DNA extraction, PCR amplification, and sequencing: Two or three specimens per colony were sequenced individually. Individual specimens were crushed in a 1.5 ml microcentrifuge tube and DNA was extracted and purified using the QIAamp DNAmicrokit (QIAGEN Inc., Valencia, CA). The mitochondrial gene Cytochrome Oxidase I (COI) was amplified in two overlapping fragments: 5' fragment with forward primer C1-J-1718 (Simon et al. 1994) and internal reverse primer C1-J-2411 (Lagos et al.

2012); 3' fragment with internal forward primer C1-N-2509 (Lagos et al. 2012) and reverse primer TL2-N-3014 (Simon et al. 1994). The nuclear gene Elongation Factor-1-α (EF1-α) the following primers were used: EF3F (Lagos et al. 2012) and EF2 (Palumbi 1996). The length polymorphism of an intron in SCP was sequenced using the primers Aph13 and Aph15 (Carletto et al. 2009a). All primers were synthesized by Invitrogen™ Corporation (Carlsbad, CA). PCR used PuReTaq™ Ready-To-Go™ PCR 0.2 ml beads (GE Healthcare UK) mixed with 20 µl of PCR-grade water, 1 µl of F and R primers at 10 µM, and 3 µl of genomic DNA solution. The thermal cycler protocol used to amplify COI and EF1-α was: 95°C 2 min (95°C 30s; 53°C 30s; 72°C 120s) 40x. For SCP, it was: 95°C 3 min (94°C 60s, 55°C 45s, 72°C 60s) 40x. PCR products were run on a 1% agarose gel for 40 min at 90 v, and visualized with GelGreen nucleic acid stain (Biotium Inc, California, USA). Most PCR products were purified using QIAquick® (QIAGEN Inc.) kit. PCR products that included the co-amplification of non-specific bands were gel purified using Zymoclean ™ gel DNA recovery kit (Zymo Research, USA). The concentration of PCR products was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). PCR products were sequenced in both directions using 3 µl of a mixture of BigDye® Terminator v3.1, dGTP BigDye Terminator v3.0, and buffer in a ratio of 2:1:1 respectively, 1.6 µl of 2 µM primer primers, differing amounts of DNA, and 1 µl of dimethyl sulfoxide (DMSO) (SIGMA-ALDRICH®, St Louis, MO). Sequencing reactions were run using the following protocol: 96°C 2 min (95°C 20s; 50°C 5s; 60°C 240s) 25x. Sequencing reactions were cleaned using Performa® DTR Ultra 96-Well Plates (EdgeBioSystems, Gaithersburg, MD) and run on ABI 3730 at the Keck Center (University of Illinois at Urbana-Champaign). Raw sequence data were examined and assembled using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI). Sequences were then aligned with Clustal X (version 2.0, 2007; Larkin et al. 2007). Three introns in EF1-α were identified and used in this study. Nucleotide sequences were deposited in GenBank (Suppl. material 1). Pairwise distances were obtained using PAUP 4.0b10 based on the Kimura two-parameter model (Swofford 2001).

The COI sequence of the *A. gossypii* neotype specimen (GU591547) and 25 EF1-α sequences of *Aphis* spp. (especially those of species closely related to *A. gossypii*) were retrieved from GenBank: EU019867, EU019869, EU019871, EU019872, EU019873, EU019874, EU019875, EU019876, EU019878, EU019879, EU358904, EU358907, EU358911, EU358915, EU358916, EU358917, EU358924, EU358926, EU358927, GU205375 and GU205376.

Phylogenetic analysis: Modeltest 3.7 (Posada and Crandall 1998) was used to select the best-fit nucleotide substitution model. Single sets of gene sequences were analyzed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2003) to execute Bayesian analyses. For single analysis, four chains were run. The number of generations was 5,000,000 with a burn-in of 250 trees and frequency sampling of 100 generations with rates equal to variable gamma as a model of substitution of nucleotides. *Rhopalosiphum maidis* (Fitch) (Aphidinae: Aphidini), and *Hyadaphis tataricae* Aizenberg and *Uroleucon helianthicola* (Olive) (Aphidinae: Macrosiphini) were selected as outgroups.

Aphid biology: Two growth chambers were used to examine various aspects of the biology of *A. monardae*, *A. gossypii*, and *A. sedi* Kaltenbach, in order to discern differences in their life cycle. Experimental plants were grown in a greenhouse in 12.7 cm diameter pots and isolated in 13.5 by 13.5 by 22.5 inches cages. Chamber A was set at 12°C and short photoperiod (8L:16D), conditions that will trigger the development of sexual morphs. Colonies of *A. monardae* on *Monarda fistulosa* L., *A. sedi* on *Hylotelephium telephium* (L.) H.Ohba, and *A. gossypii* on *Cucurbita pepo* L. and *Rhamnus cathartica* L. were exposed to these conditions for extended lengths of time. Samples of *A. monardae* and *A. sedi* were collected on a weekly basis from the host plants listed above and examined for the presence of sexual morphs. In the cages of *A. gossypii* weekly samples were taken from *R. cathartica*.

The B chamber was set at 24°C with constant illumination (24 hours) to keep colonies and test host plant specificity of the three species mentioned above. The following experiments were done in chamber B: a *Monarda fistulosa* plant infested with *A. monardae* was placed into a cage with an aphid-free *C. pepo* plant and left for a several weeks. Biweekly examination of the *C. pepo* plants was made to determine if *A. monardae* had colonized them. A *Cucurbita pepo* plant infested with *A. gossypii* was placed into a cage with aphid-free *M. fistulosa* and *H. telephium* and left for several weeks. Biweekly examination of *M. fistulosa* and *H. telephium* was made to see if *A. gossypii* had colonized them. A *Hylotelephium telephium* plant infested with *A. sedi* was placed into a cage with aphid-free *C. pepo* and left for several weeks. Biweekly examination of *C. pepo* was made to see if *A. sedi* had transferred to them. An entire tree of *R. cathartica* infested with *A. gossypii* was isolated in a 2 by 2 by 2-m walk-in cage in May of 2011 on the grounds of the South Farms of the University of Illinois (Suppl. material 1). The temperature ranged between 10 and 22 °C, http://www.isws.illinois.edu/atmos/statecli/cuweather/. Aphid-free *C. pepo*, *H. telephium* and *Glycine max* were placed into the cage to document the potential infestation of these secondary hosts under natural environmental conditions.

Results

Phylogenetic analysis

A total of 160 COI sequences from 28 species, 133 EF1-α sequences from 36 species, and 13 SCP sequences from 6 species were used in this study. After alignment and excluding the primer sites, 1,290, 1,078 and 703 bp for COI, EF1-α (including gaps and introns) and SCP were used in the analysis, respectively. COI sequence divergence between species of the *A. gossypii* species group ranged from 0.08% (between *A. gossypii* and *A. sedi*) to 3.04% (between *A. gossypii* and *A. monardae*). The sequence divergence of *A. glycines* and *A. nasturtii* (sharing a winter host plant with *A. gossypii*), as compared with the species of the *gossypii* group, ranged from 5.25% (between *A. gossypii* and *A. glycines*) to 6.97% (between *A. nasturtii* and *A. sedi*) (Table 1). The

Table 1. Range of Kimura 2 Parameter pair-wise inter- and intraspecific sequence divergence (%) for COI sequences.

	A. forbesi	***A. glycines***	***A. gossypii***	***A. monardae***	***A. nasturtii***	***A. oestlundi***	***A. sedi***
A. forbesi	0.00						
A. glycines	5.49–5.73	0.00					
A. gosyypii	6.27- 6.35	5.25–5.92	0.00–0.54				
A. monardae	6.27	5.75–5.85	2.70–3.04	0.00–0.08			
A. nasturtii	5.73–5.77	7.03–7.15	6.66–6.89	6.50–6.73	0.00–0.08		
A. oestlundi	6.35	5.67–5.85	2.37–2.57	1.57–1.81	6.57–6.68	0–0.16	
A. sedi	6.2–6.35	5.51–5.76	0.08–0.70	2.62–3.02	6.54–6.97	2.37–2.77	0.00–0.54

Table 2. Range of Kimura 2 Parameter pair-wise inter- and intraspecific sequence divergence (%) for EF1-α and SCP sequences.

	A. gossypii		***A. monardae***		***A. oestlundi***		***A. sedi***	
	EF1-α	**SCP**	**EF1-α**	**SCP**	**EF1-α**	**SCP**	**EF1-α**	**SCP**
A. gossypii	0.40–0.87	0.14–0.84						
A. monardae	0.54–0.97	1.12–1.98	0.00–0.11	0.14–0.28				
A. oestlundi	0.76–1.20	1.12–1.83	0.87–0.98	0.42–0.64	0.00	0.00		
A. sedi	0.11–0.76	0.84–1.84	0.65–0.76	1.26	0.87	1.26	0.00–0.22	0.00

sequence divergences of EF1-α and SCP are presented in Table 2. Generally, COI sequences were more conserved than EF1-α, which in turn were more conserved than SCP.

The cladograms using COI (Figure 1) and EF1-α (Figure 2) showed a high level of agreement. The COI analysis supported the monophyly of a group of species (Clade A) related to *A. gossypii*, including *A. glycines, A. nasturtii*, and a still more closely related group that are regarded as members of the *A. gossypii* complex (Clade D). Within Clade A are several supported groups: Clade H of *A. rubifolii* (Thomas) and *A. rubicola* Oestlund (PP:1.00); Clade I of *A. nasturtii* and *A. urticata* Gmelin (PP:1.00); Clade B of *A. glycines* with the *A. gossypii* complex (PP:0.99). The *A. gossypii* complex is itself well supported (Clade D, PP:0.99), and includese two groups: Clade E of *A gossypii* and *A. sedi* (PP:0.99) and Clade F of *A. oestlundi* Gillette, *A. monardae*, and several possible new species.

The dendrogram inferred by MrBayes using EF1-α (Figure 2) is congruent with that of COI for some taxa mentioned above (clade A, PP:0.99), although lack of resolution prevented recovery of a monophyletic Midwest *A. gossypii* complex. The close relationship of *A. nasturtii* and *A. urticata* is robustly supported (Clade G, PP:1.00) as is clade B (PP:0.99). Clade A in the COI analysis is polyphyletic in the EF1-α analysis and includes Asian species *A. ichigicola* Shinji and *A. ichigo* Shinji (Clade F, PP:0.97); *A. glycines* and *A. sanguisorbicola* Takahashi (Clade E, PP:0.98). Clade C is poorly supported and presents polytomies of species closely related to *A. gossypii*:

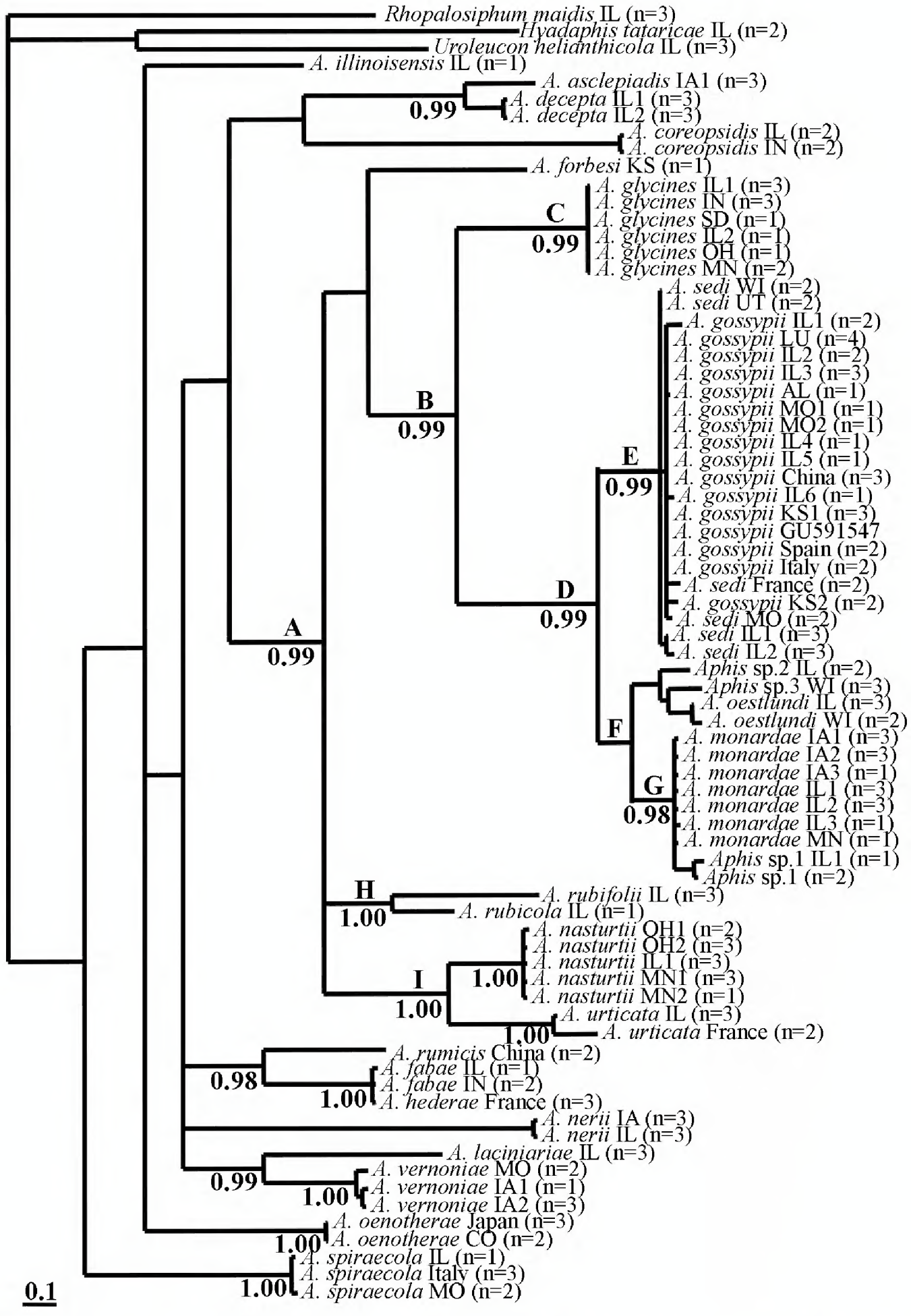

Figure 1. Cladogram inferred based on analysis of COI with MrBayes. Support values (Posterior Probabilities) are below branches. Values under 0.95 are not presented. Species names are followed by collection locality (USA: AL (Alabama), CO (Colorado), IA (Iowa), IL (Illinois), IN (Indiana), KS (Kansas), LA (Louisiana), MO (Missouri), MN (Minnesota), OH (Ohio), SD (South Dakota), WI (Wisconsin)), and number of haplotypes.

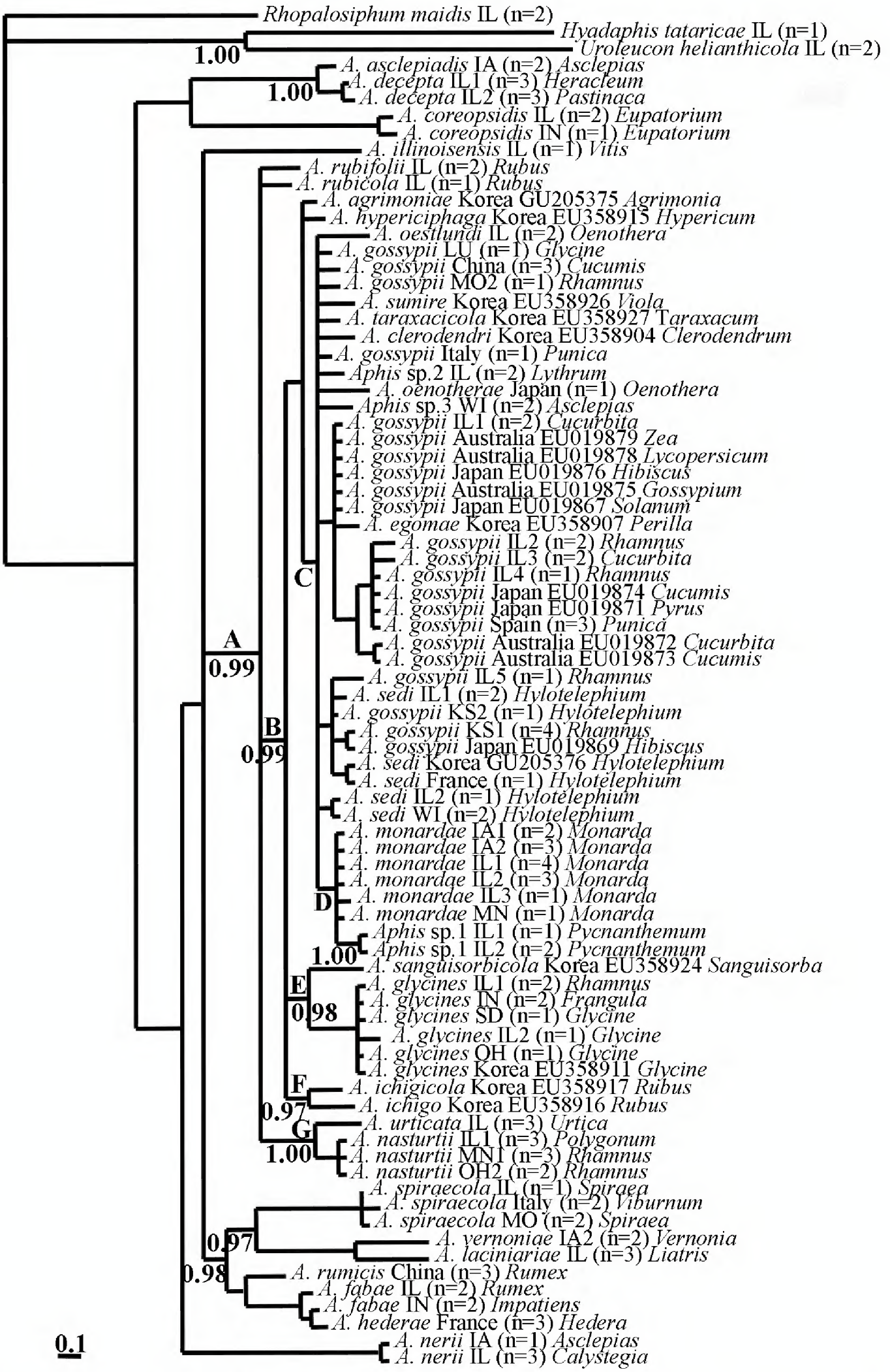
Rhopalosiphum maidis IL (n=2)
Hyadaphis tataricae IL (n=1)
Uroleucon helianthicola IL (n=2)
1.00
A. asclepiadis IA (n=2) Asclepias
A. decepta IL1 (n=3) Heracleum
1.00
A. decepta IL2 (n=3) Pastinaca
A. coreopsidis IL (n=2) Eupatorium
A. coreopsidis IN (n=1) Eupatorium
A. illinoisensis IL (n=1) Vitis
A. rubifolii IL (n=2) Rubus
A. rubicola IL (n=1) Rubus
A. agrimoniae Korea GU205375 Agrimonia
A. hypericiphaga Korea EU358915 Hypericum
A. oestlundi IL (n=2) Oenothera
A. gossypii LU (n=1) Glycine
A. gossypii China (n=3) Cucumis
A. gossypii MO2 (n=1) Rhamnus
A. sumire Korea EU358926 Viola
A. taraxacicola Korea EU358927 Taraxacum
A. clerodendri Korea EU358904 Clerodendrum
A. gossypii Italy (n=1) Punica
Aphis sp.2 IL (n=2) Lythrum
A. oenotherae Japan (n=1) Oenothera
Aphis sp.3 WI (n=2) Asclepias
A. gossypii IL1 (n=2) Cucurbita
A. gossypii Australia EU019879 Zea
A. gossypii Australia EU019878 Lycopersicum
A. gossypii Japan EU019876 Hibiscus
A. gossypii Australia EU019875 Gossypium
A. gossypii Japan EU019867 Solanum
A. egomae Korea EU358907 Perilla
A. gossypii IL2 (n=2) Rhamnus
A. gossypii IL3 (n=2) Cucurbita
C
A. gossypii IL4 (n=1) Rhamnus
A. gossypii Japan EU019874 Cucumis
A. gossypii Japan EU019871 Pyrus
A. gossypii Spain (n=3) Punica
A. gossypii Australia EU019872 Cucurbita
A. gossypii Australia EU019873 Cucumis
A
0.99
A. gossypii IL5 (n=1) Rhamnus
A. sedi IL1 (n=2) Hylotelephium
A. gossypii KS2 (n=1) Hylotelephium
B
A. gossypii KS1 (n=4) Rhamnus
0.99
A. gossypii Japan EU019869 Hibiscus
A. sedi Korea GU205376 Hylotelephium
A. sedi France (n=1) Hylotelephium
A. sedi IL2 (n=1) Hylotelephium
A. sedi WI (n=2) Hylotelephium
A. monardae IA1 (n=2) Monarda
A. monardae IA2 (n=3) Monarda
A. monardae IL1 (n=4) Monarda
A. monardae IL2 (n=3) Monarda
D
A. monardae IL3 (n=1) Monarda
A. monardae MN (n=1) Monarda
Aphis sp.1 IL1 (n=1) Pycnanthemum
Aphis sp.1 IL2 (n=2) Pycnanthemum
1.00
A. sanguisorbicola Korea EU358924 Sanguisorba
E
A. glycines IL1 (n=2) Rhamnus
A. glycines IN (n=2) Frangula
0.98
A. glycines SD (n=1) Glycine
A. glycines IL2 (n=1) Glycine
A. glycines OH (n=1) Glycine
A. glycines Korea EU358911 Glycine
F
A. ichigicola Korea EU358917 Rubus
A. ichigo Korea EU358916 Rubus
0.97
A. urticata IL (n=3) Urtica
G
A. nasturtii IL1 (n=3) Polygonum
1.00
A. nasturtii MN1 (n=3) Rhamnus
A. nasturtii OH2 (n=2) Rhamnus
A. spiraecola IL (n=1) Spiraea
A. spiraecola Italy (n=2) Viburnum
A. spiraecola MO (n=2) Spiraea
0.97
A. vernoniae IA2 (n=2) Vernonia
A. laciniariae IL (n=3) Liatris
0.98
A. rumicis China (n=3) Rumex
A. fabae IL (n=2) Rumex
A. fabae IN (n=2) Impatiens
A. hederae France (n=3) Hedera
0.1
A. nerii IA (n=1) Asclepias
A. nerii IL (n=3) Calystegia

Figure 2. Cladogram inferred based on analysis of EF1-α with MrBayes. Support values (Posterior Probabilities) are below branches. Values under 0.95 are not presented. Species names are followed by collection locality, number of haplotypes and genus of host plant.

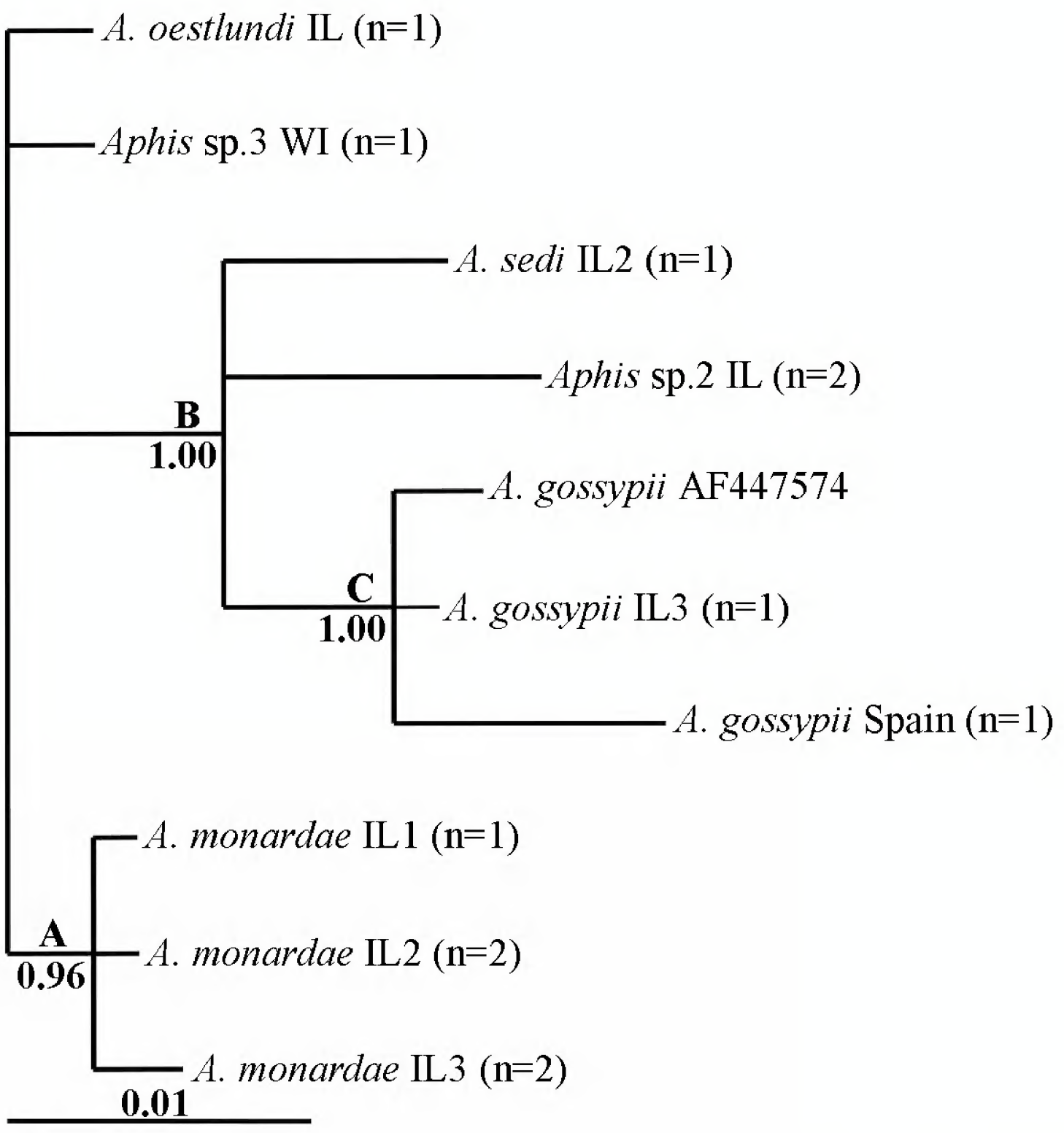

Figure 3. Inferred relationships using the SCP gene based on analysis with MrBayes. Support values (Posterior Probabilities) are below branches. Species names are followed by the collection locality (USA) and number of haplotypes.

A. sedi, *A. oenotherae* Oestlund; the Asian taxa *A. egomae* Shinji, *A. sumire* Moritsu, *A.* taraxacicola (Börner), and *A. clerodendri* Matsumura; and the North American species *A. monardae* and *A. oestlundi*.

The SCP gene was difficult to amplify and thus we only acquired sequences for six taxa. The Bayesian cladogram using SCP (Figure 3) shows two groups strongly supported: *A. monardae* (Clade A, PP: 0.96) and the group comprised of *A. sedi*, *Aphis* sp.2 and *A. gossypii* (Clade B, PP:1.00).

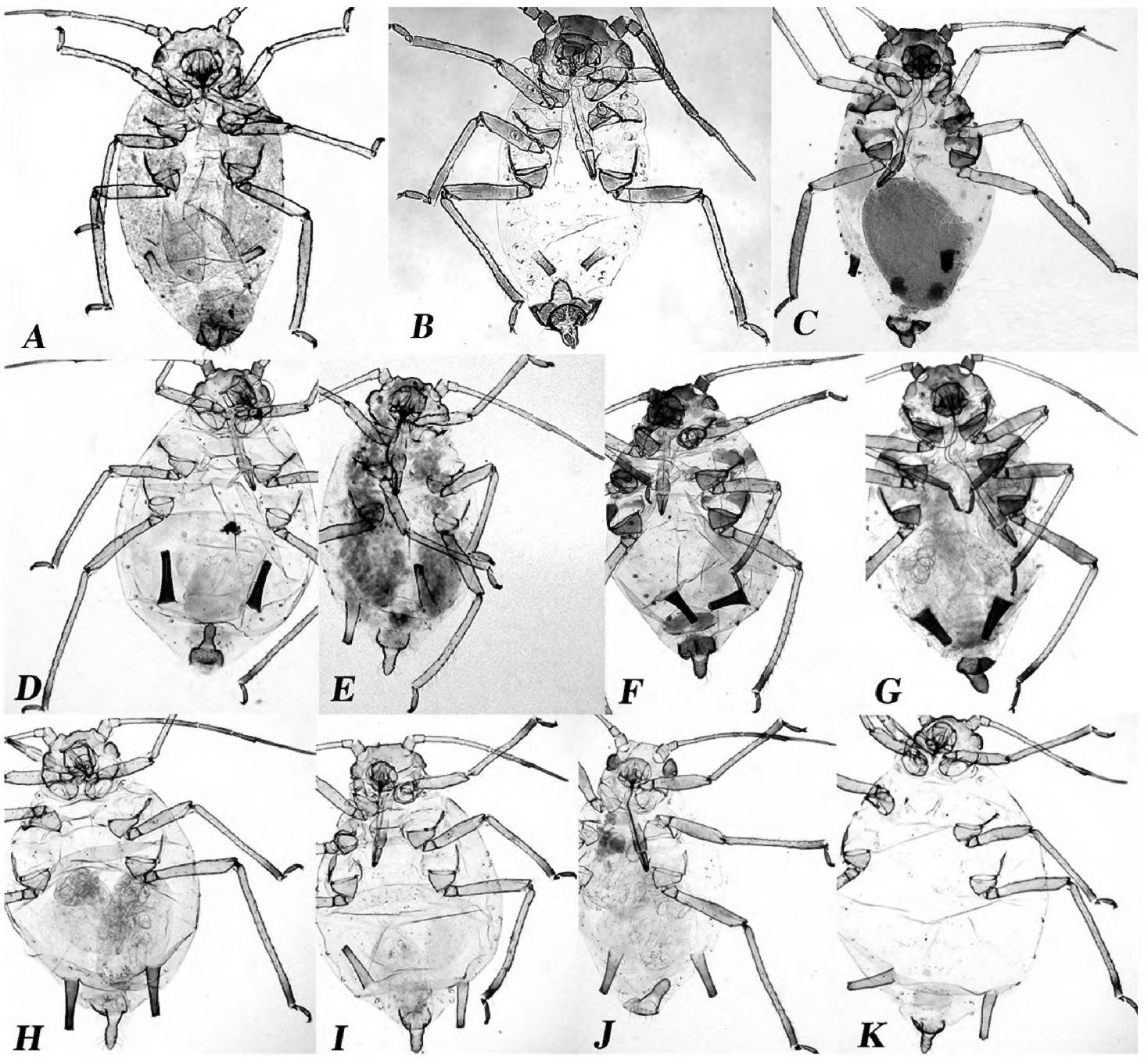

Figure 4. Habitus images of slide-mounted sexual morphs of **A** ovipara of *A. monardae* **B** male of *A. monardae* **C** ovipara of *A. sedi*, and apterous viviparae of **D** *A. gossypii* **E** *A. gossypii* **F** *A. gossypii* **G** *A. sedi* **H** *A. glycines* **I** *A. monardae* **J** *A. nasturtii* **K** *A. oestlundi*.

Biological evidence

After four weeks under conditions of reduced temperature and photoperiod, colonies of *A. monardae* reared on *M. fistulosa* produced oviparae and apterous males (Figure 4A–B). Also, sexual morphs were collected in the field (Middlefork Savanna, Lake County) at the beginning of October (Suppl. material 1). *Aphis sedi* on *H. telephium* produced oviparae (Figure 4C) but no males were found. Voucher slides of both species are deposited in the INHS insect collection with the following catalog numbers: *A. monardae*, 512858-512865; *A. sedi*, 511202-511208 and 511559-511573. In chamber A, no sexual morphs of *A. gossypii* were found after two months exposure to the low temperatures and reduced photoperiod and weekly collections on *R. cathartica*.

The outdoor experiments located at the South farms of the University of Illinois were evaluated after 25 days. Alate viviparae of *A. gossypii* were seen on *H. telephium*

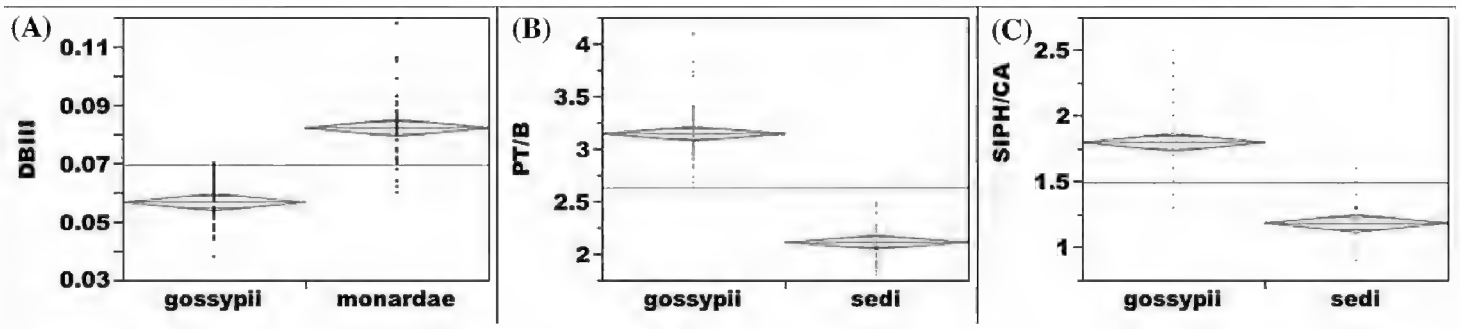

Figure 5. Analysis of variance of morphological characters useful to discriminate *Aphis gossypii, A. monardae*, and *A. sedi*. The gray line represents the median. The gray diamond represents the means and standard deviation. A 95% level indicates a significant difference. **A** distance from the base of antennal segment III to the first secondary sensorium (DBIII) between *A. gossypii* and *A. monardae* **B** ratio of length of processus terminalis (PT) to the base of last antennal segment **B** between *A. gossypii* and *A. sedi* **C** ratio of length of siphunculi (SIPH) to the length of cauda (CA) between *A. gossypii* and *A. sedi.*

and *G. max* but they did not produce offspring, however, alates that moved to *C. pepo* did produce apterous and alate viviparae. Voucher slides are deposited in the INHS insect collection numbers: 512851-512857. The colonies of *A. gossypii* reared on *C. pepo* were set in a growth chamber B where they grew rapidly. Potted *M. fistulosa* were placed in this chamber and were colonized by *A. gossypii*. Clean plants of *C. pepo* that were later exposed in the same chamber to a colony of *A. monardae* were not colonized. A colony of *A. sedi* begun with fundatrices from *H. telephium* was exposed to *C. pepo* in growth chamber B for several weeks, but the aphids did not transfer to and establish on this plant.

Comparison of *Aphis monardae* and *Aphis gossypii*

In both the COI and EF1-α analyses, *A. monardae* was readily distinguished from *A. gossypii* (Figure 1 Clade G, Figure 2 Clade D). *Aphis monardae* and *A. gossypii* are also differentiable morphologically: 1) the siphunculi of apterous morph are darker in *A. gossypii* than in *A. monardae*, and 2) and secondary sensoria on antennal segment IV are always absent in alate viviparae of *A. gossypii*, but present in *A. monardae* (Suppl. material 2). A third, novel morphological character, the distance from the base of antennal segment III to the first secondary sensorium (DBIII) in alate viviparae also separates these species consistently. In *A. gossypii,* the secondary sensoria are uniformly distributed along the segment (Figure 6B) but not in *A. monardae* (Figure 6C). The means of the distance from the base of antennal segment III to the basal margin of the first secondary sensorium of *A. gossypii* and *A. monardae* are 0.06 and 0.08 mm, respectively (Figure 5A, F ratio=152.3, df=1, P<0.0001). Evidence in support of the reproductive isolation of this species is the presence of oviparae (Figure 4A) and apterous males of *A. monardae* (Figure 4B) on *M. fistulosa* (INHS insect collection numbers: 511335-511344 and 512858-512865, respectively), as well as a COI sequence divergence of 2.7-3.04% between *A. gossypii* and *A. monardae* (Table 1).

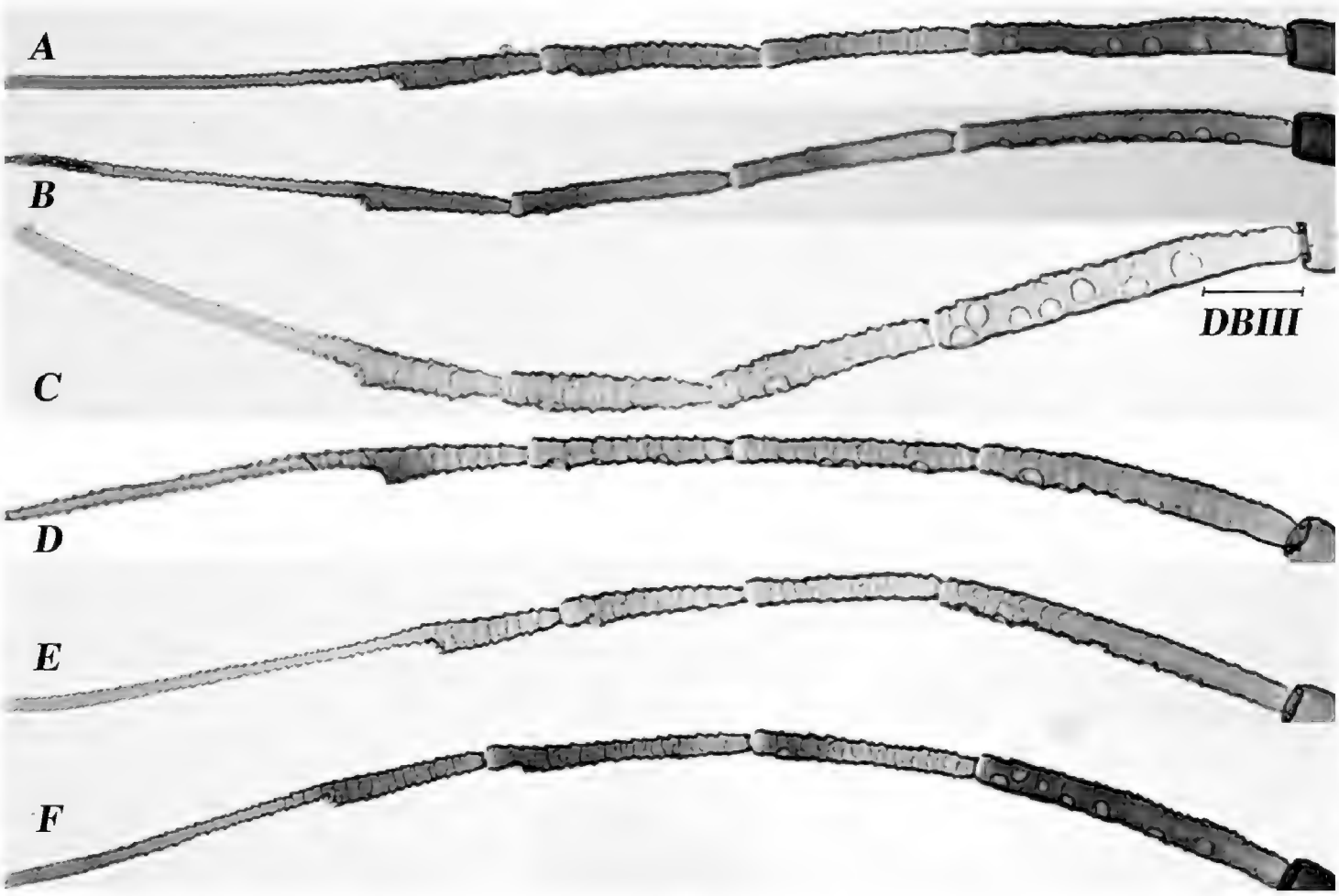

Figure 6. Antennal segments (II-VI) of alate viviparae **A** *A. glycines* **B** *A. gossypii* **C** *A. monardae*, showing distance from the base of antennal segment III to the first secondary sensorium, DBIII **D** *A. nasturtii* **E** *A. oestlundi* **F** *A. sedi*.

Redescription of *Aphis monardae* Oestlund, 1887

Diagnosis: Siphunculi of apterous morph pale, dark distally. When alive, light yellow to light green, body covered with white wax (Figure 8B). In alate viviparae: secondary sensoria on antennal segment IV present (Figure 6C). The distance from the base of antennal segment III to the first secondary sensorium (DBIII) 0.06-0.12 (0.08).

Neotype: Apterous viviparous female. USA: Minnesota; Douglas County; on *Monarda fistulosa* L.; 45.8160°N, 95.7472°W; 19.viii.2010; D. Lagos. Neotype apterous viviparous female (INHS Insect Collection 513070). Body1.4, URS 0.09, accessory setae 2, antennal segments: III 0.16, IV 0.08, V 0.09, B 0.08, Pt 0.18, LHIII 0.010, hind tibiae 0.50, HT2 0.08, width of tubercle on abdominal tergite I 0.020, width of tubercle on abdominal tergite VII 0.018, siphunculus 0.19, cauda 0.12, with 5 setae, abdominal tergite VIII with 2 setae, sub-genital plate with 3 setae on anterior part.

See Suppl. material 2 for morphological measurements of the four morphs of *A. monardae*. Additional images of *A. monardae* can be found in Lagos et al. (2014a).

Apterous viviparae (n= 40). *Color in life* (Figure 8B): Head, thorax and abdomen vary from light yellow to light green. *Color of cleared specimens* (Figure 4I): *Head:* dusky. All antennal segments pale, except the sixth throughout, which is dusky. Secondary sensoria absent. URS does not reach the hind coxae. *Thorax:* Coxae, trochanters and all femora dusky. All hind tibiae dusky and dark distally. *Abdomen:* Cauda

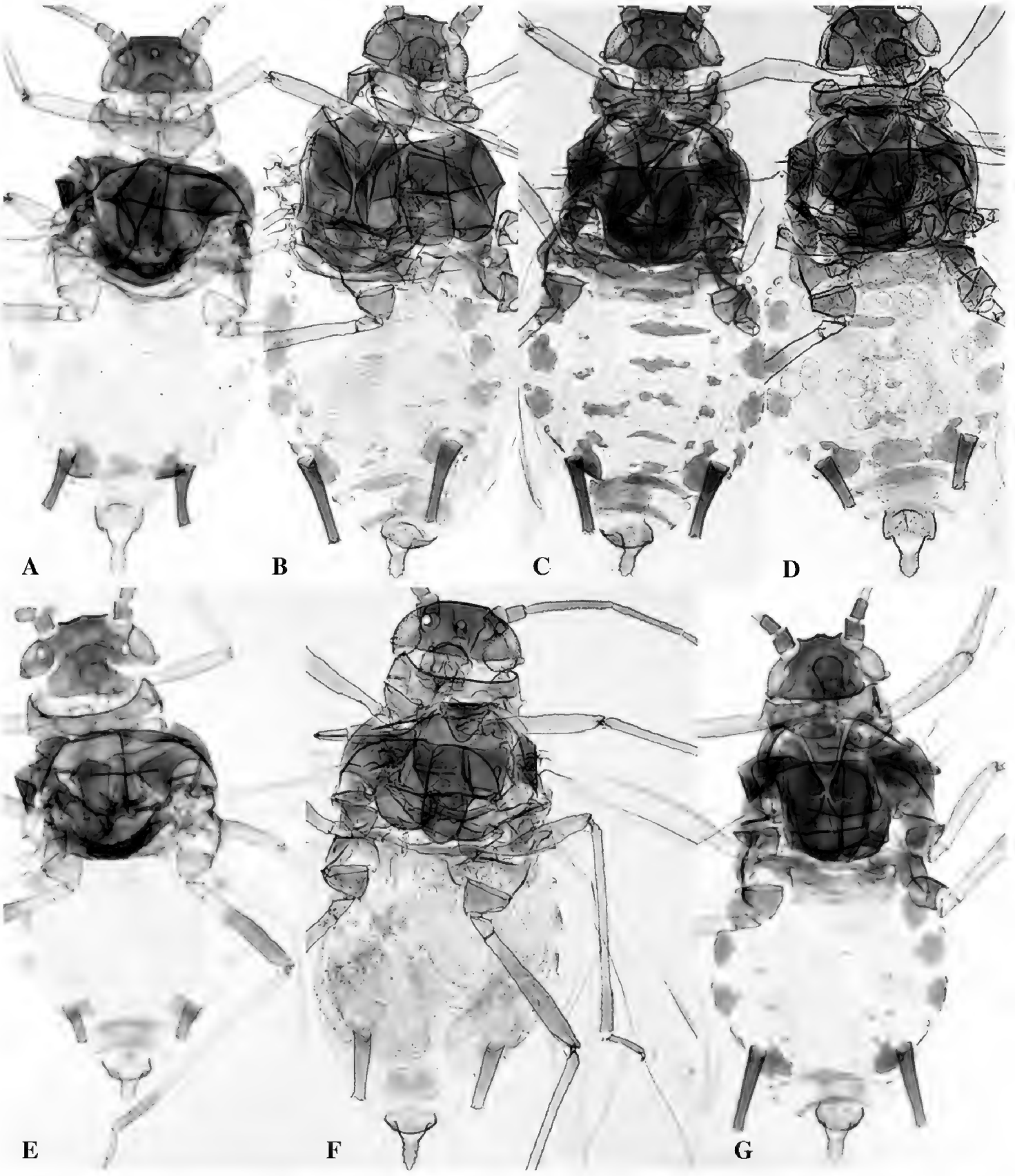

Figure 7. Body of alate viviparae. **A** *A. glycines* **B** *A. gossypii* **C** *A. gossypii* **D** *A. sedi* **E** *A. monardae* **F** *A. nasturtii* **G** *A. oestlundi*.

slightly dusky, tongue-shaped. Siphunculi dusky and dark distally, imbricated with flange. Marginal sclerites pale. Marginal tubercles only present on abdominal segments I and VII. Dorsal abdomen without sclerites. Pre and post-siphuncular sclerites. Abdominal tergite VIII with 2 setae. Subgenital plate complete, slightly dusky with 2-7 setae on anterior part. Cuticle with reticulation.

Alate viviparae (n= 59). *Color in life* (Figure 8B): Head and thorax brown. Abdomen green. *Color of cleared specimens* (Figure 7E): *Head*: dark. Antennal segments:

Figure 8. *Aphis* species of the *A. gossypii* complex. **A** Apterous vivipara of *A. gossypii* on *Rhamnus cathartica* **B** Nymphs, apterous and alate viviparae of *A. monardae* on *Monarda fistulosa* **C** Apterous ovipara of *A. monardae* **D** Nymphs and apterous male (brownish in the center of the image) of *A. monardae* **E** Nymphs and alate vivipara of *A. gossypii* on *Cucurbita pepo* **F** Nymphs and apterous vivipara of and *A. oestlundi* on *Oenothera biennis* **G** Apterous vivipara (top) and apterous ovipara (bottom) of *A. sedi* on *Hylotelephium telephium*.

first and second dark, the rest dusky. Secondary sensoria present on and III and IV. Arrangement of secondary sensoria in a single row on the distal half (Figure 6C). *Thorax:* All femora dusky except in the base. Hind coxa dark. Hind trochanters paler than coxa. Hind tibiae dark distally. *Abdomen:* Cauda pale or slightly dusky. The cauda parallel-sided with constriction near the base. Siphunculi dark throughout,imbricated with flange. Pre-siphuncular sclerites absent. Post-siphuncular sclerites dusky. Marginal sclerites pale. Marginal tubercles only present on abdominal segments I and VII. Dorsal abdomen with small transverse sclerites on VI, VII and VIII. Abdominal tergite VIII with 2 setae. Subgenital plate complete, slightly dusky, with 2-7 setae on anterior part. Cuticle without reticulation.

Oviparae (n= 26). *Color in life* (Figure 8C): *Head*: varies from light brownish to dark green. Antennal segments: first, second and ¾ of third pale yellowish, the rest dusky. *Thorax:* Coxae and trochanters pale or dusky. Fore femora dusky throughout, mid-femora dusky except at base, hind femora dark except at base. Tibiae dusky distally and tarsi dusky. *Abdomen*: Cauda dark green. Siphunculi lighter than dark green abdomen. *Color on slide and morphological characters* (Figure 4A): *Head*: Dusky without frontal setae. Antennal tubercle undeveloped. Antennae five-six segmented, shorter than body. Antennal segments: first, second, third and four pale, the rest dusky. Rostrum reaches mesocoxae. *Thorax:* Coxae and trochanters dusky. All femora dusky throughout. Tibiae and tarsi dusky throughout. *Abdomen*: Cauda dusky, parallel-sided with blunt tip and bearing 6-8 setae. Siphunculi pale, smooth with flange. Pre and post-siphuncular sclerites absent. Marginal tubercles only present on abdominal segments I and VII. Dorsum

of abdomen without sclerites. Abdominal tergite VIII with 4-8 setae. Subgenital plate dark, with 4-17 setae on anterior part. Cuticle without reticulation.

Alate male (n=17). *Color in life* (Figure 8D): *Head*: brownish. Antennae: blackish. *Thorax:* greenish. Legs light brown and tibiae distally dark as well as tarsi. *Abdomen*: Cauda dark green. Siphunculi lighter than dark green abdomen. *Color on slide and morphological characters* (Figure 4B): *Head*: dark. Antennae dark with secondary sensoria scattered on segments III, IV, and V. *Abdomen*: Cauda pale or dusky, parallel-sided with blunt tip and bearing 3-6 setae. Marginal tubercles present on abdominal segments I and VII. Dorsum of abdomen without large transverse sclerites. Male genitalia with 2 short claspers anteriorly and aedeagus centrally.

Comparison of *Aphis sedi* and *Aphis gossypii*

The distinction of *A. sedi* from *A. gossypii* is supported by phenotypic characters of specimens in collections included in Tables S1 and S2. In addition, morphological characters such as the ratio of the lengths of the processus terminalis and the base of the sixth antennal segment (Suppl. material 2, Figure 5B: F ratio=498.1, df=1, P<0.001) and the ratio of the lengths of the siphunculus and the cauda (Suppl. material 2, Figure 5C: F ratio=168.5, df=1, P<0.001) of apterous viviparae can be useful to discriminate these species. Interestingly, only oviparae of *A. sedi* reared on *Hylotelephium telephium* were collected under laboratory conditions (Figures 4C and 8G). In contrast with the morphological differences, the interspecific genetic divergences using COI and EF1-α sequences of *A. gossypii* and *A. sedi* are less than 1% (Tables 1 and 2). SCP showed greater genetic divergence between these two species, namely 0.84–1.84% (Table 2).

Comparison of *Aphis gossypii* with *Aphis forbesi*, *Aphis glycines* and *Aphis nasturtii*

These species are sometimes misidentified because they share some morphological characters on either apterous or alate morphs. Moreover, the pair-wise sequence divergences using COI sequences between *A. gossypii* and *A. forbesi* Weed, *A. glycines* and *A. nasturtii* are up to 5% (Table 1). Here we present some characters that can be useful for their discrimination. Apterous viviparae of *A. gossypii* can be differentiated from those of *A. forbesi* by the width of the marginal tubercles on abdominal segments I and VII (maximally 0.011 in *A. gossypii* and minimally 0.025 in *A. forbesi*; range for *A. gossypii* is given in Suppl. material 2), number of antennal segments and color pattern of siphunculi. Apterae of *A. gossypii* are differentiated from those of *A. glycines* by the shape of the cauda (Figures D-F, H) and the number of caudal setae, and from those of *A. nasturtii* by the absence of marginal tubercles on abdominal segments II and VI (Figure 4J). Alate viviparae of *Aphis gossypii* can be differentiated from those of *A. forbesi* by the number of secondary sensoria on III and the DBIII (Suppl. material 2), from those of *A. glycines* by the color of the hind coxae and marginal sclerites (Figure 7A) and from

those of *A. nasturtii* by the number of secondary sensoria on antennal segments III, IV and V, absence of marginal tubercles on abdominal segments II and VI, and shape of cauda (Figure 7F). More figures and morphological characters have been uploaded in Lagos et al. 2014a.

Dichotomous keys to apterous and alate viviparous females of the *Aphis gossypii* complex in the Midwest

Many dichotomous keys to subsets of *Aphis* have been written (Hottes and Frison 1931, Palmer 1952, Rojanavongse and Robinson 1977, Cook 1984, Stroyan 1984, Heie 1986, Brown 1989, García Prieto et al. 2005, Blackman and Eastop 2006) when morphological characters were not useful to discriminate between species, host plant associations have been used. Unfortunately, in the Midwest *A. gossypii* has been found on most of the host plants of other *Aphis* species included in this complex (*A. gossypii*, *A. monardae* stat. nov., *A. oestlundi* and *A. sedi*). The alternative key that we present below is based on specimens from collections made in the Midwest, and molecular data for specimens from these collections (Tables 1 and 2) supports our morphologically based identifications. Morphological data for these species is shown in Suppl. material 2. For some comparative morphometric data of European specimens of *A. gossypii* and *A. sedi* see Stroyan (1984), Heie (1986), Brown (1989) and García Prieto et al. (2005). The key is specific to Midwest collected specimens and may not be reliable in other geographic regions. It also demonstrates the difficulty of separating these closely related species using only morphological characters.

Key to apterous viviparae

1 Cauda pale, most often with constriction at midpoint, with 4–7 setae. Antennae five or six segmented. Siphunculi pale, distally dusky. Summer morphs. Polyphagous (Figure 4E) ..... ***A. gossypii***

– Cauda dusky or dark **3**

3 Siphunculi dark all throughout **4**

– Siphunculi dusky or lighter at the base 7

4 Cauda constricted **5**

– Cauda not constricted 7

5 Cauda spoon-shaped, distinctly constricted, with 4–7 setae. Ratios PT/B 2.6–4.1, SIPH/CA 1.3–2.5. Polyphagous (Figure 4D) ..... ***A. gossypii***

– Cauda slightly constricted **6**

6 Cauda slightly constricted at midpoint, with 4–5 setae. Ratios PT/B 2.0–2.7, SIPH/CA 1.5–2.2. On *Oenothera* spp. (Figure 4K) ..... ***A. oestlundi***

– Cauda elongate, parallel-sided, with acute tip and slight constriction at the base, and with 4–8 setae. Ratios PT/B 1.8–2.5, SIPH/CA 0.9–1.6. On

Hylotelephium spp. (and elsewhere recorded from *Sedum* spp. and some other Crassulaceae) (Figure 4G) ..... ***A. sedi***

7 Siphunculi lighter at the base, dusky distally. Cauda tongue-shaped, with 6–9 setae. Ratios PT/B 1.7–2.9, SIPH/CA 1.3–1.7. On *Monarda* spp. (Figure 4I) ..... ***A. monardae***

– Siphunculi dusky. Cauda tongue-shaped, with 4–7 setae. Ratios PT/B 2.6–4.1, SIPH/CA 1.3–2.5. Polyphagous (Figure 4F) ..... ***A. gossypii***

Key to alate viviparae

1 Cauda tongue-shaped, with 3–9 setae, without sclerites on dorsum abdominal segments I, II, and III. Secondary sensoria on antennal segment III (4–9), IV (0–3). DBIII 0.07–0.12 (Figure 6C). Ratios PT/B 1.9–3, SIPH/CA 1.1–1.8. (Figure 7E) ..... ***A. monardae***

– Cauda constricted, sometimes with sclerites on dorsum of abdominal segments I, II, and III **2**

2 Antenna VI PT/B 2.1–3.6. Secondary sensoria on antennal segment III (4–10) DBIII 0.04–0.07 (Figure 6B). Sometimes with transverse sclerites on dorsum of all abdominal segments (Figures 7B-C). Ratio SIPH/CA 1.1–2.3. Polyphagous ..... ***A. gossypii***

– Antenna VI PT/B 1.9–2.3. Secondary sensoria on antennal segment III (7–10) and IV (0–2) (Figure 6F). Sometimes with transverse sclerites on dorsum of all abdominal segments (Figure 7D). Ratio SIPH/CA 0.9–1.5. On *Hylotelephium* spp..... ***A. sedi***

– Antenna VI PT/B 2.2–2.9. Secondary sensoria on antennal segment III (2–8) (Figure 6E). Never with sclerites on dorsum of abdomen (Figure 7G). Ratio SIPH/CA 1.8–2.1. On *Oenothera* spp..... ***A. oestlundi***

Discussion

The analysis of different species included in this study largely corroborates the results obtained by Coeur d'acier et al. (2007), Kim and Lee (2008), Kim et al. (2010a), Kim et al. (2010b), Kim et al. (2011) and Lagos et al. (2014b). The *gossypii* complex in the North American Midwest contains the following native species, *A. oestlundi* and *A. monardae*, and the invasive species *A. gossypii* and *A. sedi*. Collection host records for *A. gossypii* show that it has been collected on *Oenothera* and *Monarda*, the host plants of the native *Aphis* species listed above (Blackman and Eastop 2006). Collection records for the native species suggest a very limited host range, in contrast with the highly polyphagous *A. gossypii*. Our results indicate that these species can be differentiated by morphological characters as well as host association. Data from this study confirms the finding of Lagos (2007) that *A. monardae* is a valid species and not a synonym of *A. gossypii* (Eastop and Hille Ris

Lambers 1976). The novel character (distance from the base of antennal segment III to its first secondary sensorium, DBIII) is useful to differentiate alate viviparae of *A. monardae* and *A. gossypii* when they are collected together in traps. The sexual morphs collected on *Monarda* under laboratory and field conditions indicate that *A. monardae* has a monoecious holocyclic life cycle. A neotype of *A. monardae* has to be designated according to the Article 75.3 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 1999). Concomitant with the redescription of the species, we here designate a neotype of *A. monardae* from the state of Minnesota on *Monarda fistulosa*. Slides deposited by O.W. Oestlund in the Insect Collection of the University of Minnesota show collection data no earlier than 1896. However, the first description of *Aphis monardae* was published in 1887 and the original description specified neither a type nor the type locality (Oestlund 1887). The comparison of apterae, alatae and oviparae of Oestlund's collections match the morphological characters of those collected recently (Suppl. material 1). Some slides made by Oestlund were remounted in 1968 so it was possible to better see the characters. For a neotype we chose a more recently collected specimen taken in Minnesota as it more clearly shows color pattern and other characters used in the redescription.

The discrimination of *A. gossypii* and *A. sedi* is clear when the aphids are alive (Figure 8). The identification problem arises when we examine samples that have lost their color by being stored in ethanol. Molecular data also are helpful. The pair-wise sequences divergences between these species using SCP are higher than for COI and EF1-α sequences (Tables 1 and 2). This marker also successfully differentiated the cryptic species *A. gossypii* and *A. frangulae* (Carletto et al. 2009a). Results obtained in this study corroborate the biological and morphological findings of Kring (1955), who found that *A. sedi* is holocyclic monoecious on *Hylotelephium*. In this study, only apterous oviparae were collected under laboratory conditions conducive to the production of sexuales (Figure 4C). Kring's morphological observations showed that the ratio of the processus terminalis to the base of the last antennal segment (PT/B), and the ratio of the length of the siphunculus to the length of the cauda (SIPH/CA), are both greater in *A. gossypii* than *A. sedi* for all morphs (Suppl. material 2). Although the above characters are useful to differentiate these species, their identification (especially the alate viviparae) is still problematic because of their similar morphology and because these ratios overlap (Figures 5B–C).

The inclusion of *A. glycines*, *A. gossypii* and *A. nasturtii* in strongly supported clades (Clade A, Figures 1 and 2) is consistent with the findings of Foottit et al. (2008) but in disagreement with those of Kim et al. (2010a). Interestingly, these three invasive species share a winter host plant, *Rhamnus* spp., but this is not the only known overwintering host for *A. gossypii*. This indiscriminate behavior, in addition to multiple species sharing winter hosts, raises the possibility of interspecies hybridization (Müller 1986, Rakauskas 2003). Hybridization may or may not be successful but should be detectable in studies of gene flow and phenotypic characterization of putative hybrids.

The species regarded here as members of the *A. gossypii* complex, *A. gossypii*, *A. sedi*, *A. oestlundi* and *A. monardae* (Clade D), exhibit interesting biological, morphological

and molecular patterns. *Aphis gossypii* has been shown to colonize numerous secondary host plants including those of closely related taxa (Stroyan 1984, Heie 1986, Blackman and Eastop 2006). Moreover, it is one of the few *Aphis* species with multiple primary host plants (Blackman and Eastop 2006). By contrast, the native taxa related to it and found in the Midwest have or are presumed to have monoecious holocyclic life cycles (see Suppl. material 1 for host plant information). *Aphis oestlundi*, *A. monardae* and *A. sedi* have wingless males, a characteristic that would contribute to the genetic isolation of these species. These sibling species possess morphological characters useful for diagnostic purposes (Suppl. material 2) and the values that support interspecific sequences divergences (Table 2) are similar to those found by Foottit et al. (2008) and Favret and Miller (2011). The identification of species related to *A. gossypii* is made more difficult because they feed on host plants that can also serve as host to *A. gossypii*. Interestingly, however, their colors in life differ and can be useful for identification. For example, *A. gossypii* is dark green or light brownish and its siphunculi are dark throughout (Figures 8A, E), although this can vary in summer dwarf specimens. *Aphis gossypii* is mostly darker than *A. monardae*, which is light yellow or green (Figures 8B, C), and *A. oestlundi* is light yellow (Figure 8F). The color of *A. sedi* is dark green (Figure 8G), like *A. gossypii*, although it has more white wax on its body (García Prieto et al. 2005).

The COI sequence divergence values obtained in this study are similar to those obtained in other studies (Cocuzza et al. 2009, Cognato 2006, Coeur d'acier et al. 2007, Foottit et al. 2008, Favret and Miller 2011, Wang and Qiao 2009). Moreover, the low pair-wise sequence divergences found between some species such as *A. gossypii* and *A. sedi* (Table 1) are consistent with those obtained by other workers such as Piffaretti et al. (2012). While COI data have been found useful to discern the phylogenetic relationships of many taxa, the use of COI sequence divergences to set cut-off points that can differentiate *Aphis* species should be used with caution, since it may lead to the misidentification of new species, a conclusion drawn by other studies for several orders of insects (Blaxter 2004, Hebert et al. 2004, Nadler 2002, Will and Rubinoff 2004, Smith et al. 2008).

Our work suggests the possible existence of three undescribed Midwestern species (*Aphis* spp. 1, 2, and 3) within the *gossypii* complex. Further studies need to be done to validate their status. It is likely that additional new species will be found within this group as material is gathered from a larger geographical area and combined molecular, morphological and biological data are used to analyze the new taxa. The use of multiple primary hosts is unusual for any species, thus lineages within the *gossypii* complex that select and limit themselves to specific hosts may be driving the speciation process within this group (Peccoud et al. 2010, Kim et al. 2011).

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Supplementary material 1

Table S1. Collection information.

Authors: Doris M. Lagos, Colin Favret, Rosanna Giordano, David J. Voegtlin

Data type: species data

Explanation note: Collection information for specimens included in this study. INHS voucher and GenBank accession numbers are for specimens originating from a specific collection.

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Supplementary material 2

Table S2. Morphological characters useful to discriminate *A. gossypii*, *A. monardae*, *A. oestlundi* and *A. sedi*.

Authors: Doris M. Lagos, Colin Favret, Rosanna Giordano, David J. Voegtlin

Data type: measurement data

Explanation note: Morphological characters useful to discriminate *A. gossypii*, *A. monardae, A. oestlundi* and *A. sedi*. For all measurements and counts the range is given and the mean is in parentheses. All measurements in mm. Abbreviations: B base of last antennal segment, CA cauda, DBIII: Distance from the base of antennal segment III to the first secondary sensorium, HT2 second hind tarsus, LHIII longest Hair on ant. segm. III, PT: Processus terminalis, SIPH siphunculi, URS ultimate rostral segment. Data of oviparae of *A. gossypii* from Kim et al. (2010a).

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